ANTIBACTERIAL SUBSTANCES PRODUCED BY MOLDS AND MOSSES

BY

IRVING R. HOOPER

B. A., Michigan State Normal College, 1941

AN ABSTRACT OF A THESIS

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN CHEMISTRY IN THE GRADUATE SCHOOL OF THE UNIVERSITY OF ILLINOIS, 1944

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CHAPTER I

A STUDY OF CLAVACIN AND CERTAIN OF ITS ANALOGS

Introduction

The discovery of penicillin[1] stimulated extensive research in the field of antibacterial compounds produced by living organisms. Wiesner[2] and Waksman[3] reported that the mold *Aspergillus clavatus* produced an antibacterial substance, which was named clavacin by Waksman. This material was not isolated but concentrates were reported to be active against Gram-negative as well as Gram-positive organisms. The compound was also reported to be stable to acid and heat, but unstable in the presence of alkali. This investigation includes the isolation of crystalline clavacin, a study of its properties and an examination of the antibacterial properties of some related compounds.

Experimental

Aspergillus clavatus was grown on a modified Czapek-Dox medium by Dr. H. W. Anderson of the Horticulture Department of the University of Illinois, who furnished large quantities of culture filtrates for this investigation. Cup-plate and cylinder-plate methods were used to assay the concentration of the antibacterial substance. Various methods of extracting and purifying clavacin were examined. Very pure solutions were obtained by a method involving ether extraction, concentration, purification of an aqueous solution by charcoal, and removal of other impurities by a partition chromatography. Crystalline clavacin was isolated in a pure state from this purified solution.

A simple method of large-scale production of the crystalline clavacin was then developed. This culture filtrates were extracted with ether continuously and impurities were removed from the ether solution by a charcoal treatment. Such purified solutions yield clavacin in a crystalline form when concentrated.

The properties and reactions of clavacin were studied and it was shown to have the formula $C_7H_6O_4$. While further investi-

gation of the structure of clavacin was in progress, Raistrick and coworkers[4] reported the properties and structure of patulin, which they had isolated from cultures of *Penicillium patulum*. They presented data showing that the compound was anhydro-3-hydroxymethylene-tetrahydro-γ-pyrone-2-carboxylic acid. From an examination of the data on the derivatives and the properties of patulin, it was apparent that patulin and clavacin were identical[5]. The properties of clavacin were in agreement with the expected properties of a compound with the formula proposed by Raistrick for patulin.

Studies were carried out on the antibacterial activity of a number of compounds with structures similar to that of clavacin. These included α-angelica lactone, β-angelica lactone, α,α,β-trimethyl-Δβγ-butenolide, dimethyl-α-angelica lactone, and methyl anhydromonocrotalate. Of these, only α-angelica lactone and dimethyl-α-angelica lactone were found to have antibacterial properties, suggesting that an enolizable hydrogen on the α-carbon atom of the butenolide nucleus is an essential for activity in compounds of this type. Both of these compounds were much weaker than is clavacin.

Summary

Clavacin was isolated from culture filtrates of *Aspergillus clavatus* in a pure crystalline state for the first time. Its properties and derivatives were studied and it has been shown to be identical with patulin. A simple method of obtaining clavacin in a pure form is described. From an examination of a few compounds with structures similar to clavacin, it is postulated that certain structural features must be present if compounds of this type are to possess antibacterial activity.

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CHAPTER II
THE ANOMALOUS VAN SLYKE REACTION

Introduction

Certain non-nitrogeneous compounds give nitrogen in the Van Slyke manometric amino nitrogen determination. Among these is chrysogenin, the pigment which occurs along with penicillin.[1] It was thought that an explanation for these anomalous values might be found in the oxidizing effect of nitrous acid. It is well known that active methylene compounds are converted to oximino compounds by the action of sodium nitrite in glacial acetic acid. Furthermore, certain oximino compounds can be converted to carbonyl compounds by the action of nitrogen dioxide or alkyl nitrites. This reaction often proceeds under anhydrous conditions, eliminating the possibility of a hydrolytic reaction. With these facts in mind, it was postulated that sodium nitrite in acetic acid might oxidize active methylene groups to carbonyl groups under the conditions of the Van Slyke reaction. The accompanying reduction of nitrous acid to nitrogen would account for the anomalous Van Slyke reaction on compounds of this type.

This investigation comprises a study of this reaction. Evidence has been obtained that the hypothesis presented above is correct.

Experimental

The reaction between various active methylene compounds or their oximino derivatives and nitrous acid was investigated. The first compound to be studied was resorcinol, which had been shown to give anomalous values in the Van Slyke determination[1] It was found that the reaction proceeds with the production of large amounts of inert gas, presumably nitrogen, when run at a temperature of 30°C. At a lower temperature (14-15°C) the production of nitrogen was much less and an unidentified material was isolated from the reaction mixture on dilution with water. The compound was shown to contain nitrogen and is believed to represent an intermediate in the oxidation of resorcinol to hydroxybenzoquinone.

The action of sodium nitrite in glacial acetic acid on two α-oximino-ketones was also studied. With ethyl α-oximino-acetoacetate, the reaction was shown to produce 50-80 per cent of the theoretical amount of nitrogen,assuming oxidation of the oxime grouping to a carbonyl group. The nitrogen was determined by the method of Natus.[2] Ethly α,β-diketobutyrate was isolated from the reaction mixture as the di-*p*-nitropheylhydrazone.

The reaction between sodium nitrite in acetic acid and α-oximino-propiophenone was shown to produce acetylbenzoyl as well as an inert gas, presumably nitrogen. When the reaction was run under pressure a fair yield of the diketone was obtained.

Summary

Evidence has been obtained which shows that the anomalous Van Slyke reaction may be caused by the oxidation of an active methylene group to a carbonyl group. Nitrogen is produced in the reaction by the reduction of the nitrous acid.

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CHAPTER III

AN ANTIBACTERIAL SUBSTANCE PRESENT IN SPHAGNUM MOSS

Introduction

Sphagum moss has been reported to exhibit antiseptic properties.[1] This investigation was undertaken to ascertain if such properties were caused by the presence in sphagnum moss of a specific antibacterial compound and if so, to study the properties and reactions of the compound.

Experimental

Sphagnum moss was first extracted with ether. It was found that concentrated ether extracts was antagonistic to bacterial growth. Other methods of isolating the antibacterial compound were investigated. It was found that a much greater yield of antibacterial substance could be obtained by extracting the moss with aqueous alkali. The alkaline extract was acidified and extracted with ether to give concentrated solutions of the active material. It was found that aqueous solutions could be purified without loss of activity by a charcoal treatment. Concentration of ether solutions yielded mainly inactive, non-crystalline precipitates, with some recovery of activity in the distillate fraction. The compound was found to be volatile in nature and acidic. It was inactive in aqueous solutions unless tested at low pH values. These facts indicated that the antibacterial substance was a low molecular weight acid, whereupon further isolation studies were discontinued.

Summary

It has been shown that sphagnum moss contains an acidic, volatile substance which inhibits bacterial growth in the undissociated form. It can be extracted from the moss by ether or more effectively by aqueous alkali. Its properties are such as to render it valueless as a practical antiseptic.

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VITA

The author was born June 16, 1921, near South Lynn, Michigan. He received his elementary and secondary education in the public schools of that region. He attended Michigan State Normal College from which he received the Bachelor of Arts degree in June, 1941. He entered the graduate school of the University of Illinois in June, 1941. He held a Teaching Assistantship in Biochemistry from September, 1941 to September, 1942 and during the summer semester of 1943. He held a University Research Fellowship in Chemistry during his second year of graduate study and a Du Pont Research Fellowship during his third year.